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Short communication

Equistatin and equinatoxin gene expression is influenced by environmental temperature in the sea anemone *Actinia equina*

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ABSTRACT

We examined the gene expression levels of equinatoxin and equistatin in the sea anemone *Actinia equina,* when reared at varying environmental temperatures for five months. Both genes were significantly downregulated at 10 °C compared to 16 °C but showed no significant change at 22 °C. This provides the first evidence of an effect of temperature on gene expression, but with no effect of increasing temperatures such as those predicted due to climate change.

The composition of venom in various conspecifics may change over time, space and physiological conditions (Tsai et al., 2001; Duda et al., 2009; Gutiérrez et al., 2010; Winter et al., 2010; Rodríguez-Ravelo et al., 2013; Chang et al., 2015). Intraspecific variations have been documented in some cnidarians (Winter et al., 2010). However, the hypothesis that venom variation may be linked to environmental temperature has not previously been experimentally tested. *Actinia equina* venom is a complex cocktail of toxins that function in prey capture, with fractions targeting potassium channels (Minagawa et al., 1998), inducing haemolysis (Maček and Lebez, 1988), and forming pores which can cause cytolysis (Athanasiadis et al., 2001). Cnidarians, such as sea anemones and jellyfish, often rely on their venom as the main means of incapacitating prey, although genes such as equistatin may be expressed elsewhere in the body if they function in multiple roles. The current study examined the influence of temperature on the expression of two protease inhibitor and toxin genes within *A. equina*. The present work describes, for the first-time, potential links between environmental temperature and gene expression in cnidarians.

Actinia equina specimens were sourced from a public aquarium (Blue Reef Aquarium, Tynemouth, UK) derived from wild, locally-collected anemones. They were held in the facility for two years using seawater sourced directly from the North Sea, therefore temperature ranges reflected the natural environment (8–13 °C in winter to a maximum of 17 °C in summer; S. Webb, personal communication). Experimental temperatures reflected the natural temperatures encountered by wild populations in this region (10, 16 °C), plus one temperature above the normal range (22 °C). A temperature of 16 °C was used as the control temperature in gene expression analysis as it represents the ambient environmental temperature at the time of collection from the aquarium facility. Anemones were reared in separate polycarbonate tanks with filtration and water delivered individually for each tank. Tanks were 28.5 × 18.5 × 12.5 cm (5 L), contained 33 ppt artificial seawater and were exposed to a 16:8 L:D cycle. No additional substrate was provided and the anemones readily adhered to the tank bottom. One animal from each tank was used as a single biological replicate in later analysis. Three true biological replicates were used (three anemones) per gene, per temperature treatment for qPCR. Additionally, three technical replicates were run for each biological sample (three replicates from each anemone), totalling nine samples per gene, per temperature treatment. Animals were fed three times per week either with live juvenile *Artemia* sp. or frozen and thawed adult brine shrimp and small krill. *A. equina* has been shown to fully digest and egest crustaceans within 12–23 h (Kruger and Griffiths, 1997). Although the South African *Actinia*, used by Kruger and Griffiths (1997) has since been reclassified as a separate species (Schama et al., 2012), we observed similar egestion rates in the *A. equina* specimens used in the current study. As such, RNA was extracted 48–72 h after feeding to prevent extraction of *Artemia* or krill RNA. Anemones were housed at experimental temperatures for five months prior to RNA extraction.

All *A. equina* target and reference gene sequences were sourced from the National Center for Biotechnology Information (NCBI) online database. There has only been limited sequencing of select genes from *A. equina,* therefore the following commonly used reference genes were chosen for primer design: 16S rRNA (Gao et al., 2011; Venkatesh et al., 2006), 28S rRNA (Xue et al., 2010; Zhong and Simons, 1999) and 5.8S rRNA (Galiveti et al., 2010; Sun et al., 2010; Shi and Chiang, 2005). We noted stable Cq values for 16S and 28S rRNA across all temperature conditions in our subsequent qPCR. Primer sequences were designed

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Table 1
Primer information. Gene name and associated forward and reverse primers designed with Primer3. T_m = melting temperature. bp = base pair. Successfully validated primers in **bold**. Multiple accession numbers represent instances where multiple partial sequences have been condensed.

Gene name	Accession number(s)	Oligo	Start (bp)	Length (bp)	T_m	GC%	Sequence	Product size (bp)
5.8S rRNA	DQ831308	Forward	13	20	58.43	50	TAACGGTGGATCTCTTGGCT	155
		Reverse	167	20	58.88	50	TGGAAACCGACACTCAGACA	
16S rRNA	KP090856.1, KP090857.1, KP090858.1, KP090860.1, KP090861.1, KP090862.1, KP090863.1, KP090866.1, KP090867.1, KP090868.1, KP090869.1, KP090870.1, KP090871.1, KP090872.1, KP090873.1, KP090874.1, KP090875.1, KP090878.1, KP090876.1, KP090879.1, KP090880.1, KP090881.1, KP090882.1, KP090883.1, KP090884.1, KP090886.1, KP090885.1, KP090887.1, KP090888.1, KP090889.1, KP090890.1, KP090891.1, KP090892.1, KP090893.1, KP090894.1, KP090895.1, KP090896.1, KP090897.1, KP090898.1, KP090899.1, KP090900.1, KP090901.1, KP090902.1, KP090903.1, KP090904.1, KP090905.1, KP090906.1, KP090907.1, KP090908.1, KP090909.1, KP090911.1, KP090912.1, KP090913.1, KP090915.1, KP090914.1, KP090916.1, KP090917.1, KP090929.1, KP090930.1, KP090931.1, KP090932.1	**Forward**	**19**	**23**	**57.11**	**39.13**	**AGTTTAGATAATGTGGGATCCGT**	**161**
		Reverse	**179**	**21**	**59.04**	**52.38**	**CCGGCTATTAACTACGGACCT**	
28S rRNA	KP065560.1, KP065559.1, KP065558.1, KP065557.1, KP065556.1, KP065555.1, KP065554.1, KP065553.1, KP065552.1, KP065551.1, KP065550.1, KP065549.1, KP065548.1, KP065547.1, KP065546.1, KP065545.1, KP065544.1, KP065543.1, KP065542.1, KP065541.1, KP065540.1, KP065539.1, KP065538.1, KP065537.1, KP065536.1, KP065535.1, KP065534.1, KP065533.1, KP065532.1, KP065531.1, KP065530.1, KP065529.1, KP065528.1, KP065527.1, KP065526.1, KP065525.1, KP065524.1, KP065523.1, KP065522.1, KP065521.1, KP065520.1, KP065519.1, KP065518.1, KP065517.1, KP065516.1, KP065515.1, KP065514.1, KP065513.1, KP065512.1, KP065511.1, KP065510.1, KP065509.1, KP065508.1, KP065507.1, KP065506.1, KP065505.1, KP065504.1, KP065503.1, KP065502.1, KP065501.1, KP065500.1, KP065499.1, KP065498.1, KP065497.1	**Forward**	**28**	**19**	**58.77**	**57.89**	**GACGAGTCGGGTTGTTTGG**	**102**
		Reverse	**129**	**20**	**59.4**	**55**	**TCCCTCACGGTACTTGTTCG**	
AeNa	AF130344	Forward	32	20	59.03	50	ACCACGAATCTGCAGAGACA	157
		Reverse	188	20	58.66	50	TACTTGGGCCGTCACTTACA	
Equinatoxin II	U41661	Forward	225	20	58.94	50	TGATGGTGCAAGTCTGTCCT	155
		Reverse	379	20	58.96	50	GGACGATATCAGAGGTGCCA	
Equinatoxin IV	AF057028	Forward	330	20	58.96	55	TGGCACCTCTGATATCGTCC	240
		Reverse	569	20	58.9	55	TCGTACATCCTCTGGTCTGC	
Equinatoxin V	U51900	**Forward**	**552**	**20**	**58.9**	**55**	**GCAGACCAGAGGATGTACGA**	**196**
		Reverse	**747**	**20**	**58.9**	**55**	**GAGGCAGCATTTCAACCGAT**	

(*continued on next page*)

Table 1 (*continued*)

Gene name	Accession number(s)	Oligo	Start (bp)	Length (bp)	T_m	GC%	Sequence	Product size (bp)
Acrorhagin	AB212067, AB212066	Forward	223	20	59.2	50	TGTCTACAGCGTCGAATCGT	207
		Reverse	449	20	59.13	50	GCAGCGTCCTTTGAACATCA	
Equistatin	AF156179	**Forward**	**366**	**20**	**59**	**55**	**CCATCGTGTAAAGCTGACGG**	**174**
		Reverse	**539**	**20**	**58.99**	**50**	**TGAATGCGCCTTGATTCGAG**	
Neurotoxin 1	EU124478	Forward	54	20	57.98	50	TGCAGATGGTAATGACGGTG	247
		Reverse	300	20	57.98	50	ACGGAATACCCCTCTTTGCT	
Neurotoxin 2-1	EU124479	Forward	239	20	58.98	55	GCCATCTTGAAAGGTAGCCG	223
		Reverse	461	20	59.17	55	TATTGCCTCTCGGTCTTGGG	
Neurotoxin 3-1	EU124482	Forward	24	20	58.7	50	ATTTGCAGCCGTATTCCTCG	157
		Reverse	180	20	59.11	55	GTTGCCACCATAACCACCTG	
Neurotoxin 4-1	EU124483	Forward	24	20	58.7	50	ATTTGCAGCCGTAATCCTCG	154
		Reverse	177	19	59.12	57.89	GTTGCCACCATAACCACCG	

using the open access software Primer3 (Table 1) for a total of ten target toxin genes (the sum total available for *A. equina*) and three reference genes. Primer sequences were then run in an NCBI BLAST search to ensure they specifically aligned only to the intended target gene. Custom primers were obtained from Bio-Rad, UK as a forward and reverse mix.

Individual, whole specimens of *A. equina* were rapidly euthanised by immersion in 70% ethanol. Anemones were of a standard size: pedal disk diameter (2–2.5 cm) and column height (1–2 cm); however, only a radial quarter was used for extraction. Each specimen was briefly towel dried, weighed, and dissected radially into quarters. One section was then weighed and homogenised with a sterile manual micro tissue homogenizer at room temperature with 1 ml TRIzol® reagent per 100 mg of tissue. All pipette tips used were sterile filter tips certified free of DNase, RNase, DNA and pyrogens. RNA was extracted following TRIzol® manufacturer's instructions and the RNA extract was further subjected to DNase treatment (Promega DNase). RNA quantity and quality were analysed using a NanoDrop™ spectrophotometer and RNA quantity was normalised prior to DNase treatment, cDNA synthesis and qPCR. cDNA synthesis was conducted from RNA samples via reverse transcription using the iScript cDNA synthesis kit from Bio-Rad, UK.

All primers were tested for validity and efficiency using endpoint PCR, agarose gel electrophoresis and the production of standard curves. Only primers for equinatoxin V, equistatin, 16s rRNA and 28s rRNA were successfully validated (correct product size observed on agarose gel in a single band, and primer amplification efficiency > 85%) and used in subsequent RT-qPCR analysis. Unsuccessful primer sequences are presented (Table 1). Whilst 5.8S rRNA produced the expected sized band, an amplification product was also noted from the negative control sample suggesting primer dimer formation; AeNa also produced a correct sized single gel band but had a low amplification efficiency of 41%. All other genes did not produce any bands on agarose gel. As Equinatoxin V is a member of a gene family with at least five members, only three of which have been sequenced, amplification of multiple copies within the family may have occurred due to similar sequences (see sequence alignments, supplementary material 1). Therefore, amplification and quantification using Equinatoxin V primers, will subsequently be generalised as equinatoxin expression.

The RT-qPCR samples were prepared using the iTaq™ Universal SYBR® Green Supermix (BIO-RAD) following manufacturer's instructions. A dilution series was also made (to calculate amplification efficiency) with nuclease free water which was dispensed in duplicate. Amplification efficiency was calculated as:

$$E = 10^{-1/slope}$$

where E = amplification efficiency of qPCR and *slope* = the slope of the standard curve of SYBR Green fluorescence.

A master mix containing all reagents except a cDNA template was then prepared on ice and 9 µL aliquots were pipetted into qPCR plate wells. A single well for each gene was loaded with nuclease free water added to the master mix to be run as a no template control to monitor contamination and primer-dimer formation. All other wells had either a positive or negative sample of cDNA template (template synthesised with or without reverse transcriptase) added.

RT-qPCR conditions were as follows: Initial denaturing step of 5 min at 95.0 °C, second step of 95.0 °C for 15 s, and 55 °C for 30 s. At this point a plate read was recorded and the cycle returned to step two. This was repeated 45 times. Finally, a melt curve analysis was conducted; 65–95 °C in 0.5 °C increments at 5 s per step, during which plate reads were recorded.

To quantify the levels of gene expression, the fold change was calculated by the double delta Cq method, using the Cq values for each gene using the following equation:

$$Fold\ change\ ratio = \frac{(E_{target})^{\Delta Cq_{target}(control-sample)}}{(E_{ref})^{\Delta Cq_{target}(control-sample)}}$$

where E = efficiency value of PCR, E_{target} = efficiency value of the target gene, E_{ref} = averaged efficiency value of the reference genes, control = the optimum temperature for the sea anemones (16 °C), and sample = altered temperature for the sea anemones.

Identified outliers were only removed from within technical triplicate replicates, which still maintains triplicate biological samples for robustness of data. Outliers were identified by analysing the difference in Cq value between the Cq target and Cq ref. for each replicate at a specific temperature and identified (as an outlier) where the difference in Cq (ΔCq) was obviously greater in one replicate compared to the others (M. Edwards, Newcastle University, Personal communication, 2017). Outliers such as these did not affect overall if a gene was shown to upregulate or downregulate (Fig. 1), they did however influence the significance of the statistical tests by affecting the standard deviation and were thus omitted from analysis as removing outliers via various methods is standard in qPCR normalisation (Goni et al., 2009; Debes et al., 2016; Manzine et al., 2018; Smith et al., 2018). 28S rRNA and 16S rRNA were processed as reference genes. Fold change data were $\log_2$ transformed. Log fold change data for equistatin and equinatoxin (10 °C and 22 °C compared to 16 °C) were normally distributed (Anderson-Darling, $P < 0.05$). One-sample t-tests were conducted to determine whether gene expression at experimental temperatures was different to normal (optimum temperature), defined as a log fold change of 0.

Equinatoxin expression was significantly downregulated at 10 °C ($t = -112.51$, $p = 0.005$; Fig. 1), with no statistical difference between the 22 °C and 16 °C treatments ($t = 1.60$, $p = 0.356$). Equistatin was also significantly downregulated at 10 °C ($t = -12.75$, $p = 0.006$; Fig. 1), but there was no significant downregulation at 22 °C ($t = -3.04$, $p = 0.202$). It should also be noted that cnidome analysis was conducted and a significant interaction between temperature and nematocyst type only documented in the pedal disk (Scheirer-Ray-Hare Test, $H = 8.528$, $P < 0.05$), with spirocysts present only at 10 °C, other

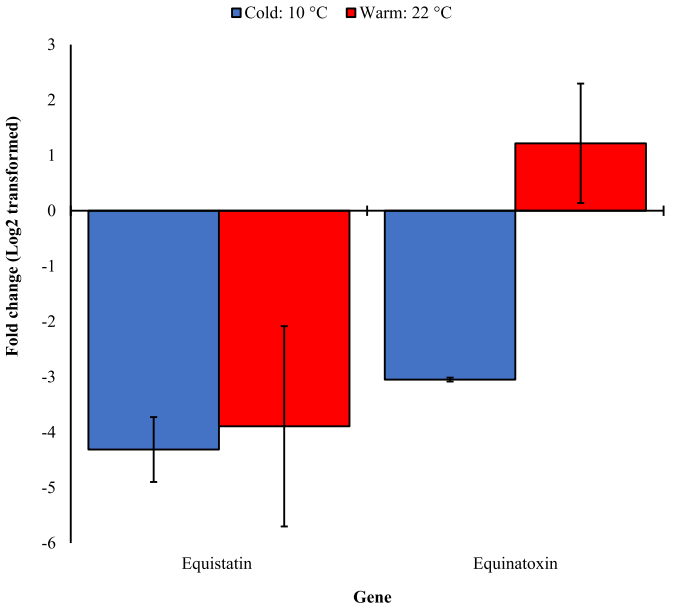

Fig. 1. Mean fold change of gene expression (log_2 transformed) (± SD) for sea anemones cultured at cold and warm temperatures. Calculated against two reference genes and an optimum temperature (16 °C) as the control for double delta Cq analysis.

temperatures only containing B-mastigophores. Unfortunately, a low sample size prevented robust interpretation of this data.

Using RT-qPCR we determined that environmental temperature can significantly influence the expression of toxin encoding genes, with the low temperature treatment leading to a relative downregulation of expression in both genes investigated. Understanding the biological roles of each toxin allows us to hypothesise as to the ecological significance of such gene expression changes. Equistatin is a cysteine and aspartic proteinase inhibitor isolated from whole body extracts of *A. equina* (Štrukelj et al., 2000). The function of equistatin is currently unresolved; however, protease inhibitors in sea anemones have been theorised to function in a number of potential roles: 1) protecting the animal from the proteases of their prey; 2) preventing the injected toxins from degradation by the proteases of envenomed animals; 3) regulating food digestion and preventing enzymatic or symbiotic organism self-digestion; and, 4) functioning in prey paralysis (Mourão and Schwartz, 2013). *A. equina* does not host symbiotic algae (zooxanthellae) (Harland et al., 1990), so a role preventing digestion of symbionts is unlikely. As equistatin has a dual nature (inhibiting both cysteine and aspartic proteases) (Mourão and Schwartz, 2013), it may function in multiple roles, however equistatin has been shown to be toxic in multiple artificial diet toxicity bioassays (Gruden et al., 1998; Outchkourov et al., 2003; Valizadeh et al., 2013) and is therefore likely function in prey capture. Thus, exposure to long term temperature decrease may impact the animal's ecology, whilst temperature increase is unlikely to impact the anemone's ability to capture prey.

Equinatoxins form transmembrane pores to permeate cell membranes, ultimately resulting in cell lysis (Kristan et al., 2009), and predator-prey interactions have been suggested to be the main function (Anderluh et al., 1999) - an idea supported by the lethality of equinatoxins to *A. equina*'s prey, such as molluscs, fish and small crustaceans (Kristan et al., 2009). Expression levels for all copies within a multigene family can vary between body parts in sea anemones (Prentis et al., 2018). Possession of such isotoxins is suggested to facilitate a broader range of prey capture (Anderluh et al., 1999), which has been similarly noted in other venomous organisms (Olivera et al., 1990). Anderluh et al. (1999) postulates equinatoxin copies may be regulated in response to season, prey availability or between specimens, and within the current study we demonstrate equinatoxin was significantly downregulated when acclimatised to 10 °C (Fig. 1). Upregulated expression was also noted with a temperature increase; however, this was not statistically significant. As noted above, the primers used in this study are likely to have amplified multiple genes within the equinatoxin family, our results therefore reflect the generalised expression of the equinatoxin family throughout the whole anemone; however, as each of these toxins are very similar and function in the same way (pore formation) within prey capture, the ecological consequences of these gene expression changes remains the same. Up or downregulation of the equinatoxins as a whole could certainly impact the animal's ecology. Again, we note that temperature increases due to climate change are unlikely to impact the anemone's use of this toxin, as no relationship between temperature increase and gene expression was observed; however, long term temperature decrease may influence the function of equinatoxin in prey capture.

Some studies have documented seasonal variation in venom composition, e.g. the content of melittin and phospholipase A2 in Africanised honeybee venom; however, the variation could not be correlated with climate (temperature or humidity; Ferreira et al., 2010). Similarly, the potency of box jellyfish (*Chironex fleckeri*) venom varies seasonally, although venom variations were also found within the same season but between geographic locations (Winter et al., 2010). This suggests that whilst seasonal venom variation is not attributable to short-term temperature differences, the current work evidences that long-term temperature differences within the animal's current temperature range do influence venom. Using similar reasoning, these results demonstrating that toxin gene expression is affected by environmental temperature provides a potential explanation for geographic location affects on venom composition in a variety of animals (Gutiérrez et al., 2010; Rodríguez-Ravelo et al., 2013; Tsai et al., 2001; Chang et al., 2015; Duda et al., 2009; Winter et al., 2010). The temperatures at each geographic location may well be a determining factor in interspecific venom composition. We would encourage future research to test the effect of metabolic rate on expression of these genes, since seasonal variations may be due to a balance between metabolic expenditure, the cost of venom production and the likely prey capture success. Evidence suggests metabolism is reduced or increased respectively in some cold- and warm-acclimatised cnidarian species (Mangum et al., 1972). However, there is some disagreement in the literature regarding metabolic cost of venom production; some studies document high costs (McCue, 2006; Pintor et al., 2010; Nisani et al., 2012; Morgenstern and King, 2013), whilst more recently, Smith et al. (2014) concluded that metabolic cost of venom production is negligible compared to routine homeostatic maintenance.

Recent work has shown that cnidarians acclimated to a water table can exhibit reduced nematocyst density and hemolytic activity in comparison to wild specimens (Ben-Ari et al., 2018), which the study concluded was a result of reduced predation pressure and differing prey availability when held in a controlled laboratory setting. It should therefore be noted that the patterns of gene expression change presented in the current study, may well be a reduced effect of what may manifest in wild anemones under the same temperature conditions. We found no difference in expression levels between the ambient summer environmental temperature (16 °C) and an elevated temperature of 22 °C, suggesting that in these temperate North Sea anemones, no immediate effects of temperature increase due to climate change would be anticipated on expression of the equistatin and equinatoxin genes. However, species that live closer to their thermal tolerance limits may have different responses, and further research focussing on short term daily temperature changes may be beneficial.

Ethical statement

This research, approved by the Newcastle University Ethics in Research Committee, was conducted in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, and the ARRIVE guidelines.

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Transparency document

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.toxicon.2018.08.004.

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